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Influence of Caffeine and Chondroitin Sulfate on Swine Sperm Capacitation and *In Vitro* Embryo Production*

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ABSTRACT

***Background*:** The establishment of an *in vitro* production (IVP) of embryo in swine allows the generation of embryos with the same quality as *in vivo* produced embryos with less costs and time. In order to achieve successful fertilization under normal circumstances *in vivo*, mammalian spermatozoa must first undergo capacitation and then acrosome reaction. The purpose of this study was compared the efficacious of IP/CFDA fluorescence and Coomassie Blue G (CB) staining to detect capacitated sperm cells in refrigerated and fresh semen. Morever, it was investigated the efficacious of caffeine and chondroitin sulphate to promote *in vitro* sperm capacitation and in vitro embryo produced (IVP) of swine embryos.

***Materials, Methods & Results*:** A sperm-rich fraction from ejaculate was obtained using the gloved-hand method and the gel-free fraction was separated using sterile gauze. The semen was diluted in BTS at a final concentration of 1.5 x 10^8 cells/mL. The sperm suspension was incubated for 2 h at 25°C, refrigerated and maintained for 1 h at 15-18°C (refrigerated group) or used immediately (fresh group). Sperm capacitation was assessed by IP/CFDA fluorescence and CB staining for both fresh and refrigerated semen. For PI/CFDA evaluation, a final solution containing 1.7 mM formaldehyde, 7.3 mM PI and 20 mM CFDA in 950 µL saline was prepared. In the dark, 40 µL PI/CFDA final solution was added to 10 µL semen and after 8 min, slides were analyzed on epifluorescence microscopy. For CB evaluation, sperm cells were fixed in 4% paraformaldehyde for 10 min and centrifuged twice at 320 x g in ammonium acetate pH 9 for 8 min. A smear was made and stained with 2.75 mg/mL CB in solution containing 12.5% methanol, 25% glacial acetic acid and 62.5% water, for 2 min. The smear was washed in running water, air dried and sealed with Permount®, diluted 2:1 in xilol to avoid staining oxidation. Our results showed that refrigeration did not affect sperm capacitation and comparing staining methods, the PI/CFDA combination was more efficient to detect capacitated sperm, when compared to CB staining. In experiment 2, we evaluated the effect of different incubation time (1 - 5 h) with chondroitin sulfate and caffeine on sperm capacitation. For *in vitro* fertilization, oocytes were obtained from slaughterhouse ovaries. Oocytes with a thick and intact *cumulus oophurus* layer and cytoplasm with homogenous granules were selected for *in vitro* maturation for 44 h. According to the results of experiment 2, it was used for *in vitro* fertilization refrigerated semen was capacitated with 50 µg/mL chondroitin sulfate for 2 h or capacitated with 5 µg/mL caffeine for 3 h. Six hours after insemination, *cumulus oophorus* cells were mechanically removed and oocytes were washed and incubated in microdrops of culture medium. Embryo development after fertilization with sperm capacitated with caffeine or chondroitin sulfate was evaluated on days 3, 5 and 7 of culture. No differences were observed in days 3 or 5 of *in vitro* culture. However, it was observed an increase on blastocyst rate on Day 7 of culture when caffeine was used as the capacitor agent.

***Discussion*:** Molecular basis of sperm capacitation is still poor understood. Sperm capacitation can occur *in vitro* spontaneously in defined media without addition of biological fluids. We observed that sperm capacitation increased as incubation period enlarged and it was observed using Coomassie blue G and PI/CFDA for fresh semen and for refrigerated semen. It can be concluded that the cooling of semen did not change their pattern of sperm capacitation and this is best assessed by IP/CFDA than by CB. In addition to the use of caffeine in sperm capacitation produces more blastocysts than the chondroitin sulfate after *in vitro* fertilization.

Keywords: sperm, PI/CFDA, Coomassie Blue G, chondroitin sulfate, caffeine, swine.

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INTRODUCTION

Sperm capacitation includes reorganization of membrane proteins, metabolism of membrane phospholipids, reduction in membrane cholesterol levels, changes in sperm motility (hyperactivation) to undergo acrosome reaction [14].

Actually, caffeine has been used to induce sperm capacitation in a majority of porcine IVF systems [3]. Caffeine is an inhibitor of cyclic nucleotide phosphodiesterase, resulting in an increase in intracellular cAMP, stimulating capacitation and spontaneous acrosome reaction of boar spermatozoa [3].

The porcine follicular fluid contains various glycosaminoglycans and its major constituent was a chondroitin sulfate produced by the granulosa cells. It is highly probable that chondroitin sulfate can promote capacitation of pig sperm [15].

The combination of IP/CFDA is able to provide information about functional aspects of sperm cell due to their molecular characteristics. The CFDA is an onfluorescent ester that is hydrolyzed after membrane penetration, resulting in formation of a highly fluorescent, membrane impermeable green fluorophore. In the other hand, PI is a bright red, nucleic acid-specific fluorophore [4].

The Coomassie Blue G staining is used to evaluate acrosome reaction. Acrosome-intact sperm stained darkly near the apical portion of the sperm head, the location of the sperm acrosome. Acrosome-reacted sperm exhibited very faint or no staining in the region of the acrosome [8].

The purpose of this study was to investigate the efficacious of caffeine and chondroitin sulphate to promote *in vitro* sperm capacitation and IVP of swine embryos. Moreover, it was compared the efficacious of IP/CFDA fluorescence and CB staining to detect capacitated sperm cells.

MATERIALS AND METHODS

Preparation of spermatozoa

Four mature boars (2-5 years of age) were used for semen collection; all animals received 2 kg/day of commercial diet and water *ad libitum*.

A sperm-rich fraction from ejaculate was obtained using the gloved-hand method and the gel-free fraction was separated using sterile gauze. The fresh semen was diluted in BTS (Beltsville Thawing Solution)[1] at a final concentration of 1.5 x 10^8 cells/mL. The sperm suspension was incubated for 2 hours at 25°C, refrigerated and maintained for 1 h at 15-18°C.

Diluted or refrigerated semen aliquots (0 or 24 h) were centrifuged in BTS at 400 x g for 8 min. Progressive motility was evaluated at 0 and 24 h after refrigeration. Only ejaculates presenting at least 70% motility were used in our work.

Experiment 1: *Comparison of PI and CFDA fluorescent probes and Coomassie Blue G staining for sperm capacitation evaluation*

For sperm capacitation evaluation, semen samples were washed at 320 x *g* in 1 mL of fertilization medium (Talp Stock), and the pellet containing spermatozoa was resuspended at 1 x 10^6 cells/mL in fertilization medium. Aliquots were stained with propidium iodide (PI)[2], carboxyfluorescein diacetate (CFDA)[2] and coomassie blue G[2].

For PI/CFDA evaluation, a final solution containing 1.7 mM formaldehyde, 7.3 mM PI and 20 mM CFDA in 950 µL saline was prepared. In the dark, 40 µL PI/CFDA final solution was added to 10 µL semen and after 8 min, slides were analyzed on epifluorescence microscopy (510-560 filter / 400x magnification). The experiment was replicated five times and two-hundred sperm cells were evaluated in each slide.

For coomassie blue G evaluation, sperm cells were fixed in 4% paraformaldehyde[2] for 10 min and centrifuged twice at 320 x *g* in ammonium acetate[2] pH 9 for 8 min. A smear was made and stained with 2.75 mg/mL Coomassie Blue in solution containing 12.5% methanol[2], 25% glacial acetic acid[2] and 62.5% water, for 2 min. The smear was washed in running water, air dried and sealed with Permount®[3], diluted 2:1 in xilol[2] to avoid staining oxidation. The experiment was replicated five times and two-hundred sperm cells were evaluated in each slide under light microscopy (400 x magnification).

Experiment 2: *Effect of caffeine and chondroitin sulfate in different periods of sperm capacitation*

The induction of sperm capacitation was performed with 5 µg/mL caffeine[2] or 50 µg/mL chondroitin sulfate[2]. Sperm cells (3 x 10^6 cells/mL) were added to 1 mL of fertilization medium (Talp Stock) suppplied with one of the capacitor agents. Spermatozoa were incubated for 5 h at 39°C in an atmosphere of 5% CO_2 in air for both capacitor agents and control group (with no capacitor). Spermatozoa were evaluated with PI/CFDA every 60 min. The most efficient method

observed in experiment 1 would be used in experiment 2 to evaluate sperm capacitation.

Experiment 3: *Effect of caffeine and chondroitin sulfate on sperm incubation before in vitro fertilization*

Abattoir-derived porcine ovaries were transported to the laboratory at 25-28°C. Follicles (2 to 5 mm of diameter) were aspirated using an 18 gauge needle attached to a 10 mL syringe, and follicular fluid was transferred into 50 mL conic tubes for sedimentation. The oocyte pellet was placed in a petri dish and examined under stereomicroscope. Oocytes with a thick and intact *cumulus oophurus* layer and cytoplasm with homogenous granules were selected for *in vitro* maturation (IVM). The medium used for oocyte maturation was North Carolina State University-23 (NCSU23) supplemented with 10% (v/v) porcine follicular fluid (PFF), 0.6 mM cysteine[2], 10 IU/mL hCG[4] and 10 IU/mL eCG[4] (Chorulon and Folligon, respectively). Porcine Cummulus-oocyte complexes were cultured in maturation medium (NCSU23) placed in a CO_2 incubator maintained at 39ºC in a humidified atmosphere of 5% CO_2 in air. After culturing for 22 h, COCs were washed three times and cultured in hCG- and eCG-free NCSU23 medium for another 22 h.

According to experiment 2 results, the shortest times of incubation with the highest rate of sperm capacitation would be chosen for fertilization procedure. Refrigerated semen was capacitated with 50 µg/mL chondroitin sulfate or 5µg/mL caffeine, both in fertilization medium in a CO_2 incubator maintained at 39ºC in a humidified atmosphere of 5% CO_2 in air. After incubation, semen was centrifuged at 320 x g for 5 min in 1mL fertilization medium and the pellet resuspended to a final concentration of 2 x 10^6 cells/mL for oocyte insemination.

Six hours after insemination, *cumulus oophorus* cells were mechanically removed from oocytes; oocytes were washed and incubated in 90 µL microdrops of culture medium NCSU23 supplemented with 4 mg/mL BSA[2].

All embryos remained at NCSU23 medium supplemented with 4 mg/mL BSA for 5 days (morulae stage); after Day 5, they were transferred to NCSU23 medium supplemented with 10% de FCS[5] (fetal calf serum) where remained until hatching; embryo development was evaluated every 48 h. The experiment was replicated five times using 180-200 oocytes per treatment.

Statistical analysis

Data were analyzed by least-squares analysis of variance using the General Linear Models (GLM) procedure of SAS[6]. The completely randomized block deign (block= boar) had a 2 x 2 factorial arrangement of treatments: 2 capacitors type (caffeine or chondroitin sulfate) and 2 cooling times (0 and 24 h). Time was analyzed as repeated measures over time. The mathematical model included main effects and all interactions. All main effects were considered fixed. Tukey test was performed to determine differences between levels of individual treatments.

RESULTS

Experiment 1: *Comparison of PI and CFDA fluorescent probes and Coomansie Blue G staining for sperm capacitation detection*

Comparing staining methods, the PI/CFDA combination was more efficient (P <0,05) to detect capacitated sperm, when compared to Coomassie blue G (CB) staining for both fresh (CB – 31.5% and PI/CFDA - 47.7%) or refrigerated semen (CB - 32.6% and PI/CFDA – 47.5%). For these reason, PI/CFDA were chosen to evaluate the effect of capacitor agents and incubation periods for fresh and refrigerated semen. Our results showed that refrigeration did not affect sperm capacitation.

Since our laboratory have a routine animal handling and no difference was found between fresh or refrigerated sperm capacitation, it was decided to use refrigerated semen in experiments 2 and 3. Furthermore, the advantage in refrigerating semen is to keep it viable for longer period of time allowing its use in an appropriated time.

Experiment 2: *Effect of caffeine and chondroitin sulfate in different periods of sperm capacitation*

Using PI/CFDA probes to evaluate sperm capacitation, the control group showed an increase of sperm capacitation between 1 and 2h of incubation, but was it not observed for 2, 3, 4 and 5h of incubation. In the group capacitated with caffeine, it was observed an increase of sperm capacitation during incubation and after 3h incubation the highest rates were reached. In chondroitin sulphate group it was also observed an increase of sperm capacitation during all incubation

periods; although only after 4h incubation the highest rates were reached (Table 1).

When evaluating the effect of each capacitor agent for the same incubation period, it was observed that chondroitin sulfate showed best results within 1, 3 and 5h incubation in comparison with other groups. The capacitated group with caffeine showed similar increase as the control group (Table 1).

Table 1. Means (%) of capacitated sperms by caffeine or chondroitin sulfate evaluated by fluorescent probes propidium iodide (PI) and carboxyfluorescein diacetate (CFDA) combination in different periods of refrigerated semen incubation.

	1h	2h	3h	4h	5h
Control	25.6 ^{Aa}	46.6^{Ba}	52.5^{Ba}	59.0^{Ba}	53.9^{Ba}
Caffeine	34.5^{Aa}	35.9^{ABa}	51.0^{BCa}	56.6^{Ca}	57.9^{Ca}
Chondroitin sulfate	52.5^{Ab}	61.5^{Aa}	63.7^{Ab}	62.4^{ABa}	75.1^{Bb}

Different superscript letters (A - C) in each line present statistical significant differences ($P < 0.05$). Different superscript letters (a and b) in each column present significant difference ($P < 0.05$).

Experiment 3: *Effect of caffeine and chondroitin sulfate on sperm incubation before in vitro fertilization*

Embryo development after fertilization with sperm capacitated with caffeine or chondroitin sulfate was evaluated on days 3, 5 and 7 of culture. No differences were observed in days 3 or 5 of *in vitro* culture. However, it was observed an increase on blastocyst rate on Day 7 of culture when caffeine was used as the capacitor agent (Table 2).

DISCUSSION

Sperm viability is related to many characteristics such as motility, vigor, morphology and structural compounds (head, acrosome, middle piece and tail) and metabolic activity. Membrane fluidity alterations interfere on its integrity such as lipid compounds alterations change polyunsaturated fatty acid oxidation leading to catabolism process. Laboratorial tests have been used to

Table 2. Means (%) of embryo development after fertilization with refrigerated semen capacitated by caffeine or chondroitin sulfate, evaluated on days 3, 5 and 7 or culture.

	Day 3		Day 5		Day 7	
	Caffeine	Chondroitin sulfate	Caffeine	Chondroitin sulfate	Caffeine	Chondroitin sulfate
2 cells	11.2	11.4	5.6	7.5	3.6	6.8
4 cells	22.2	18.2	14.6	8.3	14.8	8.5
8 cells	24.6	17.1	14.7	13.0	11.8	10.2
Morulae	-	-	21.0	21.5	19.2	10.2
Blastocyst	-	-	-	-	21.9^{A}	5.3^{B}
Degenerated	4.0	7.9	5.2	7.9	7.6	7.9
Non fertilized	38.7	42.4	38.1	42.1	36.4	42.6

Different superscript letters (A and B) in each line present statistical significant differences ($P < 0.05$).

evaluate structural integrity and sperm functional activity. Although all studies carried out intending to predict in vitro sperm fertilization ability, there is no single test that is capable to really predict it [2,4,12].

Many researchers groups used staining methods to find out sperm viability and quality [6-8]. Classical laboratory tests for seminal quality assessment is questionable because such tests rely mainly on physical and biochemical evaluations made on a relatively small population of sperm cells. Results from those tests are averages and do not take into account variations within cell populations. This type of variability can be overcome by microscopic evaluations in which individual spermatozoa are evaluated [4].

Under our study conditions, the best method for sperm evaluation was PI/CFDA probes as they allow better sperm visualization (capacitated or not) and also for being a rapid and easy method when compared to Coomassie blue G staining for both fresh and refrigerated semen.

Our results showed that refrigeration did not affect sperm capacitation. It is well established that boar spermatozoa are particularly susceptible to cold shock when cooled below 15°C. On the other hand, if semen is held at room temperature in seminal plasma for several hours the spermatozoa acquire resistance to cold shock [13]. This susceptibility might be related to the lipid composition affecting the fluidity of the plasma membrane but not the fertilizing capacity of the cells [9]. The reasons for this sperm cold shock resistance could have other hypothesis than only differences in plasma membrane lipid composition and inter-species variation [10].

Molecular basis of sperm capacitation is still poor understood. Sperm capacitation can occur *in vitro* spontaneously in defined media without addition of biological fluids [16]. Our results are in accordance to this study. We observed that sperm capacitation increased as incubation period enlarged and it was observed using Coomassie blue G and PI/CFDA for fresh semen and for refrigerated semen.

Serum albumin and energy substrates present in the capacitation media are believed to function during *in vitro* capacitation as a sink for removal of cholesterol from sperm plasma membrane increasing its fluidity. However, it has not been established whether cholesterol removal represents the only function of serum albumin, because little is known about the mechanisms of action and consequences of cholesterol removal on sperm membrane as well as its relation to capacitation. Some studies demonstrated that high density proteins, found on follicular fluid and oviduct, are able to bind to cholesterol [16].

Embryo development after fertilization using sperm capacitated with caffeine showed an increase on blastocyst rate on Day 7 of culture when compared with chondroitin sulfate. In most porcine IVF systems sperm cells are exposed to caffeine for capacitation induction before oocyte insemination. It is known that caffeine enhances sperm motility, stimulates sperm capacitation and spontaneous acrosome reaction. Nevertheless, acrosome reaction induced by caffeine may be related to the high incidence of polyspermic fertilization in porcine IVF [3]. Caffeine may be a necessary supplement for some boars [5]. Recent studies have shown a positive effect of caffeine on sperm capacitation. It was observed an increase in the penetration rate and in the efficacious of fertilization when using sperm co-incubated with caffeine for capacitation [1]. Therefore, preliminary studies for each male should be done to achieve maximum efficacious in porcine IVF.

Bovine sperm treated with chondroitin sulfate fertilize more oocytes than sperm incubated only with control medium [11]. Interestingly, in our study, chondroitin sulfate improved fertilization rate but did not increase blastocyst rates. No evidence of detrimental effect of chondroitin sulfate was found in literature, suggesting that more studies are needed to know if it can damage some sperm structure or function that do not prevent fertilization but block embryo development.

CONCLUSIONS

It can be concluded that the cooling of semen did not change their pattern of sperm capacitation and this is best assessed by IP / CFDA than by CB. In addition to the use of caffeine in sperm capacitation produces more blastocysts than the chondroitin sulfate after *in vitro* fertilization.

SOURCES AND MANUFACTURERS

[1]Beltsville Thawing Solution- IMV®, São Paulo, Brazil.
[2]Sigma-Aldrich Chemicals, St. Louis, USA.
[3]Fisher Scientific, Pittsburgh, USA.
[4]Intervet International B.V., Boxmeer, The Netherlands.
[5]Gibco, Carlsbad, USA.
[6]SAS institute Inc., Cary, USA.

Declaration of interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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